Chromatin containing CENP-A and α-satellite DNA is a major component of the inner kinetochore plate

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The pathway of molecular interactions leading to kinetochore assembly on mammalian chromosomes is unknown. Kinetochores could be specified by structural features of centromeric satellite DNA [1–3] or by specific DNA sequences, analogous to budding yeast centromeres, interspersed in centromeric satellite DNA arrays [4,5]. Alternatively, kinetochores could be epigenetic structures that replicate without strict dependence on DNA sequence [6–8]. We purified kinetochore-associated chromatin from human chromosomes by immunoprecipitation of CENP-A, a centromere-specific histone H3 homologue located in the inner plate of the kinetochore [6,9,10]. Hybridization and DNA sequence analyses of cloned kinetochore DNA fragments revealed α-satellite as the predominant sequence associated with CENP-A. A major site of micrococcal nuclease digestion was identified by mapping the termini of α-satellite clones, suggesting that the inner kinetochore plate contains phased arrays of CENP-A–α-satellite nucleosomes. These experiments demonstrate for the first time that complex satellite DNA is a structural component of the kinetochore. Further, because complex satellite DNA is evolutionarily unconserved, these results suggest that molecular recognition events necessary for kinetochore formation take place at the level of DNA conformation or epigenetic mechanisms rather than DNA sequence *per se.*

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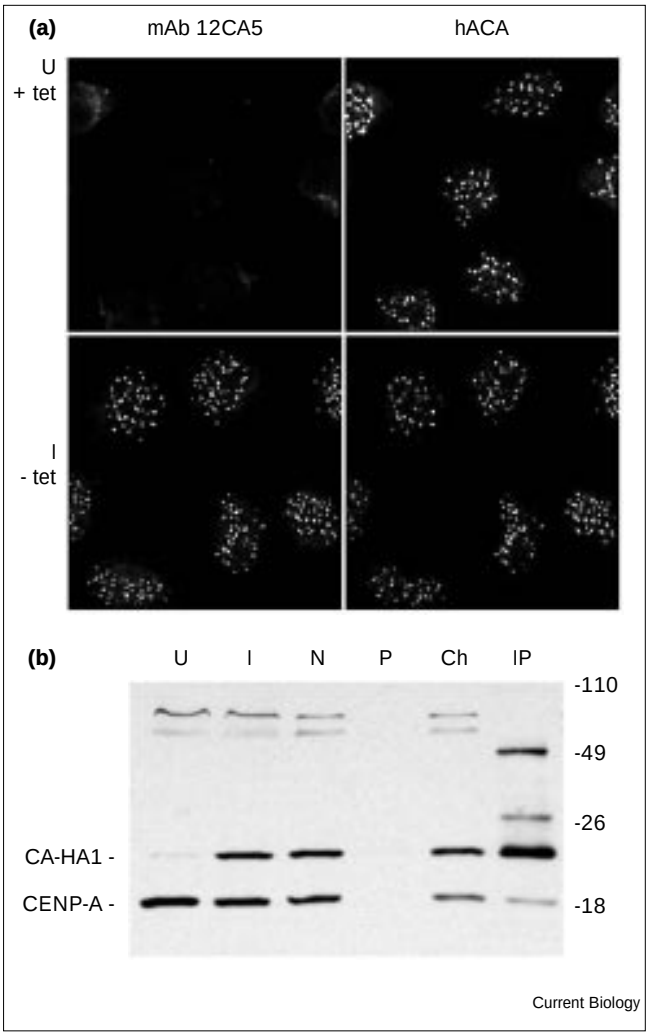
Results and discussion

The inner plate of the kinetochore contains three known components: the proteins CENP-A and CENP-C and DNA [6,11,12]. CENP-A is a homologue of the nucleosomal protein histone H3 [9] and is found in nucleosome-like particles [13]. We therefore reasoned that kinetochore-associated DNA could be isolated by purification of chromatin containing CENP-A. Centromeric chromatin was labeled in HeLa cells by expression of an epitope-tagged form of human CENP-A (CENP-A-HA1) that targets faithfully to centromeres (Figure 1a). Chromatin was prepared from labeled nuclei by micrococcal nuclease digestion and centromeric chromatin was released by mild sonication as judged by solubilization of >90% of the CENP-A (Figure 1b). Epitope-labeled chromatin was immunoprecipitated using monoclonal antibody (mAb) 12CA5, resulting in isolation of CENP-A-HA1 associated with endogenous CENP-A, as previously reported [14]. An efficient cloning strategy was developed to isolate DNA fragments that co-precipitated with CENP-A immune complexes. Preliminary DNA sequence analyses of randomly selected clones revealed an apparent enrichment of α-satellite DNA in CENP-A-associated DNA libraries (data not shown). *Alu* sequences were the next most abundant, followed by a few other interspersed repetitive elements and random DNA sequences. Enrichment of α-satellite DNA is significant as it is the most abundant DNA sequence at human centromeres [1].

To determine if α-satellite DNA or other DNA sequence classes are specifically enriched in CENP-A-associated chromatin, we used two methods. First, mixed probes representing $3–6 \times 10^4$ independent clones were prepared from chromatin-associated DNA libraries and used for Southern blot analysis of human genomic DNA (Figure 2a). DNA isolated from CENP-A chromatin hybridized to a family of DNA fragments ranging from around 340 base pairs (bp) to > 3.5 kilobase pairs (kb), separated by intervals that are multiples of 170 bp, reminiscent of α-satellite DNA (Figure 2a, IP1, IP2). This hybridization pattern was highly similar to that obtained with a mixed α-satellite probe (Figure 2a, α-sat). A control DNA probe prepared from unfractionated chromatin libraries hybridized as a broad smear similar to that observed when human genomic DNA is used as a probe (Figure 2a, Ctl1). We conclude that α-satellite DNA co-purifies with CENP-A chromatin through immunoprecipitation.

Our second approach to characterizing CENP-A-associated DNA was to sample libraries for hybridization analysis with probes for α-satellite, *Alu* and satellite III sequences (Figure 2b). α-Satellite sequences comprised approximately 40–60% of CENP-A chromatin-associated DNA fragments, but only approximately 2% of control sequences. This corresponds to an enrichment of 10–20-fold over the estimated 3–5% abundance of α-satellite in the human genome [15]. However, as CENP-A occupies only a fraction of the α-satellite domain [6], the actual degree of enrichment is probably higher. In contrast, *Alu* sequences, estimated to comprise roughly 10% of the

Figure 1



Labeling and immunoprecipitation of CENP-A-associated chromatin. (a) CENP-A-HA1 expression was induced in a stably transfected tetracycline-regulatable cell line [14]. In uninduced cells (U), CENP-A-HA1 expression was undetectable as probed with mAb 12CA5 (left). The right-hand image shows uninduced cells stained with a human anti-centromere serum (hACA) that detects endogenous CENP antigens. Following induction (I), CENP-A-HA1 expression (left) is co-localized with endogenous CENP antigens (right). (b) Immunoprecipitation of CENP-A chromatin. CENP-A-HA1 (CA-HAI) expression is shown in cell extracts of uninduced (U) and induced (I) cultures and in nuclei purified following induction (N). Chromatin from isolated nuclei was solubilized by micrococcal nuclease digestion and mild sonication; lanes labeled P and Ch are insoluble and soluble fractions, respectively. Essentially all CENP-A-HA1 was recovered in the supernatant. The soluble chromatin fraction was immuno-precipitated with mAb 12CA5, yielding a purified CENP-A chromatin fraction (IP). Fractions were resolved by SDS–PAGE and immunoblotted using a human anti-centromere serum. Bands in the IP lane above CENP-A-HA1 are heavy and light chains of mAb 12CA5. Methods were as in [14].

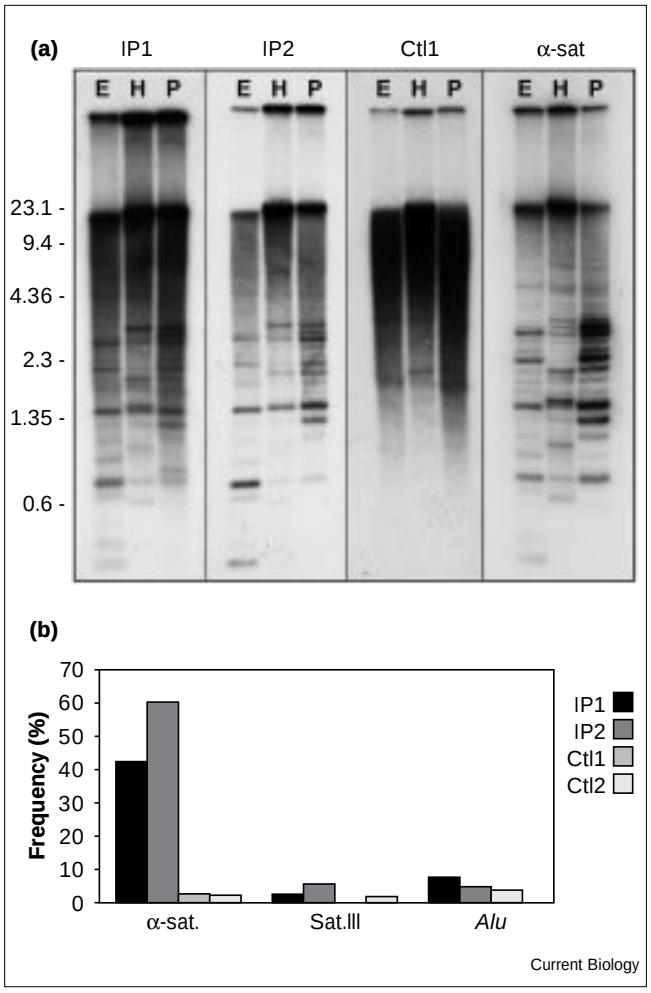
human genome, are found at a level of approximately 4–7% in CENP-A libraries, clearly not enriched. Satellite III, a pericentric simple satellite, was found at a frequency of about 4% in CENP-A-associated libraries, slightly greater than its estimated 1.5% abundance in the genome [16]. This could represent noise in the immunoprecipitation or, alternatively, a *bona fide* association of CENP-A with satellite III, perhaps at the edges of the inner kinetochore plate. Although this experiment cannot exclude specific sequence classes from the kinetochore, it suggests that most, if not all, of the CENP-A in human chromosomes is associated with α-satellite DNA.

Previous experiments used micrococcal nuclease digestion to show that bulk α-satellite DNA is packaged into preferentially phased nucleosomal arrays with a 170 bp repeat [17]. We reasoned that immunoprecipitated α-satellite DNA fragments may contain information regarding the phasing of nucleosomes on kinetochore-associated α-satellite DNA. The termini of individual fragments were mapped by sequence determination onto a 342 bp α-satellite dimer sequence (Figure 3) [15]. One 7 bp region centered at position 154 stands out as a hotspot for micrococcal nuclease digestion, containing 16% of identified termini. This region is within approximately 10 bp of that observed for the major phase on bulk α-satellite DNA [17]. It is also directly adjacent to the CENP-B box, the site of CENP-B binding to α-satellite DNA [18], consistent with the proposal that CENP-B acts as a boundary element to promote nucleosomal phasing [19,20]. Thus, the kinetochore inner plate consists, at least in part, of CENP-A chromatin containing phased arrays of α-satellite nucleosomes.

If α-satellite DNA is a *bona fide* kinetochore component, our libraries should contain representatives of all human chromosomes. Randomly selected α-satellite clones obtained by CENP-A immunoprecipitation were sequenced and characterized using BLAST [21] to examine the chromosomal origins of CENP-A-associated kinetochore DNA (Table 1). Although we cannot unambiguously establish the chromosomal origins of each α-satellite DNA fragment without *in situ* hybridization, the known features of chromosome-specific α-satellite DNA families [15] strongly support the idea that the chromosomes or chromosome families listed in Table 1 represent the most likely origins of the corresponding kinetochore DNA fragments. Representative α-satellite sequences from all but three human chromosomes were found in a sample of 56 clones. Taken together with the observation that CENP-A is a marker for active centromeres [6], we suggest that CENP-A chromatin is an obligate component of functional kinetochores on human chromosomes.

Experiments in several species point to the idea that centromere function is specified by epigenetic determinants that act independently of DNA sequence [6–8,22]. The experiments reported here demonstrate for the first time

Figure 2



α-Satellite is the most abundant class of CENP-A-associated DNA. (**a**) Southern blot analysis. DNA was purified from CENP-A immunoprecipitates and cloned into 3′dT-tailed vector pCR2.1 (Invitrogen) after treatment with Taq polymerase to add complementary 3′dA tails. Probes prepared from two separate immunoprecipitation experiments (IP1 and IP2), from total solubilized chromatin (Control, Ctl1) and from PCR-derived genomic α-satellite DNA (α-sat) [23] were hybridized to replicate filters containing human genomic DNA digested with *Eco*R1, *Hin*dIII or *Pst*1 (E, H, P, respectively). CENP-A-associated DNA hybridized to a family of genomic DNA fragments with an underlying 170-bp periodicity in size distribution, similar to that observed for α-satellite DNA. (**b**) Immunoprecipitated DNA libraries were picked into 384-well plates and replica plated onto nylon filters for hybridization analysis. Filters were hybridized with probes for genomic α-satellite DNA, *Alu* sequences and satellite III. The chart plots the percentage of positive colonies observed for each probe in four different libraries. IP1 (*n* = 392) and IP2 (*n* = 380) are two separate immunoprecipitation libraries, Ctl1 (*n* = 156) is a library generated from a mock immunoprecipitation and Ctl2 (*n* = 188) was generated from total solubilized chromatin before immunoprecipitation (*n* is the number of independent clones assayed in each experiment). α-Satellite DNA is clearly the major component of CENP-A-associated DNA.

Table 1

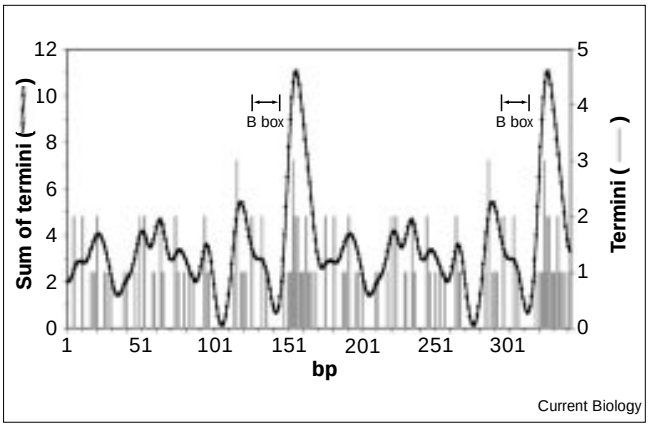
CENP-A-associated α-satellite chromosomal origins.

Chromosome	Clone	Sequenced length (bp)	GenBank locus	Smallest sum probability *P* (*N*)
1, 5, 19	ovip2o3	208	HUMSATAC	1.9e-58
2	ovipp3	177	HUMSATAA	6.9e-53
4	ovipo8	179	HSASP384	6.5e-63
6	ovip412	129	HSPA23G2	6.0e-2
7	ovipb3	142	HUMSATA7C	4.5e-22
8	ovipg11	202	HUMD8Z2AA	3.2e-59
9	ovip315	108	HUMSATALP	4.8e-38
10	ovipm3	186	HSASP1005	2.8e-52
11	ovipm2	157	HUMRSAKPA	2.3e-28
13	oviprg1	153	HUMAREPAA	4.1e-51
13,14,21	ovip420	137	HSASTRA2	7.4e-31
16	ovipc5	197	HUMSATD	3.9e-68
17	ovipg6	186	HUMSATEA	4.4e-65
18	ovipj12	192	HUM18ASA	1.5e-44
20	ovipg3	173	HUMSN2A	2.7e-42
13,21	ovipg5	158	HSAREP21	1.7e-57
X	ovip2j11	152	HSALPREP2	5.8e-54

The sequences of 56 independent CENP-A-associated α-satellite DNA fragments were compared to the non-redundant nucleotide sequence database using the BLAST server at NCBI [23]. The highest scoring hit for each chromosome is shown.

that complex satellite DNA, typified by human α-satellite DNA [16], is a structural component of the kinetochore. Previous work from our laboratory has shown that human CENP-A can recognize and assemble at kinetochores in other, non-primate, mammalian species that show similar organization of complex satellite DNA at centromeres but lack detectable DNA sequence homology [9]. Coupled with the demonstration that CENP-A can assemble at neocentromeres that lack detectable α-satellite DNA [6], these results argue against a simple DNA sequence recognition model for assembly of kinetochore chromatin. Rather, these results suggest that molecular recognition events necessary for kinetochore formation take place at the level of DNA conformation or epigenetic mechanisms rather than DNA sequence *per se*. CENP-A expression is uncoupled from bulk histone sysnthesis, with mRNA levels peaking in the G2 phase of the cell cycle after normal S-phase-dependent histone synthesis [14]. This points toward a distinctive mode of replication for kinetochore chromatin and identifies a candidate mechanism for such an epigenetic process. It is possible that over evolutionary time, protein–protein interactions at the level of the chromatin fiber have replaced direct DNA sequence recognition as a means of recruiting kinetochore components onto the chromosome surface. This could account

Figure 3



Termini of sequenced α-satellite fragments reveal nucleosome phasing. The positions of the termini of 56 cloned fragments were determined with reference to a 171-bp consensus α-satellite DNA sequence [15]. Termini are plotted with the *x*-axis representing a 342-bp α-satellite dimer, to avoid edge effects imposed by the arbitrary origin of the 171-bp monomer. Individual termini are plotted in histogram form (grey bars). Data are also plotted as the sum of termini over a 7-bp window, smoothed by averaging (black line). The position of the CENP-B box is indicated (B box). The predominant peak at 154(/325) is within 12 bp of the predominant micrococcal nuclease digestion site determined for bulk α-satellite chromatin [17].

for the relaxed DNA sequence requirements and apparent epigenetic components of centromere/kinetochore function in animal cells.

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